AD_____

MIPR NUMBER: 95MM5544

TITLE: Effect of Levonorgestrel (NORPLANT) on the Immune Regulation of Bone Morphogenesis in Calvarial Cultures from the Laboratory Mouse (Mus muscularis)

PRINCIPAL INVESTIGATOR: David W. Craft, MAJ; Arlynn G. Raez, MAJ; Carol A. Lapp, Ph.D.

CONTRACTING ORGANIZATION: Dwight David Eisenhower Army Medical Center
Fort Gordon, Georgia 30905

REPORT DATE: October 1995

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1995	Final (1 Dec 94 - 30 Sep 95)

4. TITLE AND SUBTITLE
Effect of Levonorgestrel (NORPLANT) on the Immune Regulation of Bone Morphogenesis in Calvarial Cultures from the Laboratory Mouse (Mus muscularis)

5. FUNDING NUMBERS
95MM5544

6. AUTHOR(S)
MAJ David W. Craft, MAJ Arlynn G. Raez, Carol A. Lapp Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Dwight David Eisenhower Army Medical Center
Fort Gordon, GA 30905

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Steroid sex hormones such as estrogen and progesterone affect gingival and osseous tissues and can mediate cytokine production. When administered as oral contraceptives, these hormones affect bone morphogenesis and mediate periodontal disease.[3,4,7,9] Norplant[R], the subdermal contraceptive implant, continually releases a synthetic progestin, levonorgestrel, for five years.[10,11] In order to assess the impact of levonorgestrel on bone cells, murine calvarial cell cultures were harvested, grown to confluence and treated with levonorgestrel, progesterone and estrogen. The majority of the cells grown in these bone cell cultures were phenotypically and morphologically characteristic of an osteoblast. In a group of cultures treated with levonorgestrel, bone cells secreted significantly lower amounts of IL-1β-stimulated IL-6 versus control cultures ($p < 0.05$). Similarly, cultures treated with progesterone secreted significantly lower amounts of IL-1β-stimulated IL-6 versus control cultures ($p < 0.05$), but significantly higher amounts than those treated with levonorgestrel ($p < 0.05$). In a group of cultures treated with the two progestins and estrogen, IL-1β-stimulated IL-6 levels indicated a significant interaction between the hormones ($p < 0.05$). These results suggest that both levonorgestrel and progesterone when used in combination with estrogen may partially inhibit the anti-resorptive effect of estrogen through an increase in IL-6 secretion.

14. SUBJECT TERMS
Cytokines, Levonorgestrel, Osteoblast, Bone

15. NUMBER OF PAGES
18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to *stay within the lines* to meet optical scanning requirements.

Block 1. Agency Use Only *(Leave blank)*.

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	- Contract	PR	- Project
G	- Grant	TA	- Task
PE	- Program Element	WU	- Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

- DOD - See DoDD 5230.24, "Distribution Statements on Technical Documents."
- DOE - See authorities.
- NASA - See Handbook NHB 2200.2.
- NTIS - Leave blank.

Block 12b. Distribution Code.

- DOD - Leave blank.
- DOE - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.
- NASA - Leave blank.
- NTIS - Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only)*.

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

MC Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

MC In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

29 Sep 95

PI - Signature Date

TABLE OF CONTENTS

EFFECT OF LEVONORGESTREL (NORPLANT) ON THE IMMUNE REGULATION OF BONE MORPHOGENESIS IN CALVARIAL CULTURES FROM THE LABORATORY MOUSE (MUS MUSCULARIS)

Arlynn G. Ráez, Carol A. Lapp and David W. Craft

INTRODUCTION

Recent advances in immunobiology have increased the understanding of the mechanisms of host immune responses in periodontal disease. Although bacteria are considered to be the main local factor responsible for periodontal pathogenesis, investigators have identified many different mediators of this process. For example, the role of cytokines in human diseases is a rapidly developing area of study, with interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF) apparently being the most actively involved in periodontal disease.[1,2] Studies also support the concept that altered levels of steroid sex hormones have a strong influence on the host immune reaction to inflammation.[3]

Steroid sex hormones have direct effects on gingival and osseous tissues and can mediate cytokine production as well. It is well documented that gingival inflammation is exaggerated during puberty and pregnancy, which is a time that represents a marked increase in the hormone levels of women. Other studies have also shown increased gingival inflammation and hyperplasia in women taking oral contraceptives.[3,4,5] Oral tissues are more exposed to the free (unbound) circulating hormone (from saliva and blood) when compared to other tissues (ie. muscle), therefore the oral tissues are more vulnerable to the effects of hormonal change.[3] It has been suggested that there is an immunosuppressive effect from steroid hormones, which results in a decrease in the host's immune response and contributes to the destruction of periodontal tissue.[6]

It has been demonstrated that steroid sex hormones affect inflammation through their actions in the function of polymorphonuclear leukocytes (PMN) chemotaxis[4], as well as by regulation of the circuitry of cytokine action that controls bone remodeling.[2,7] Endogenous sources for the alteration of the total level of circulating sex steroids can be multiple. Decreases in these levels can either be from decreased production (i.e. menopause), increased metabolic breakdown of these hormones, or increased production of carrier proteins. Increased levels on the other hand, can be due to increased production from usual sources and/or tumor sources. The most common reason for excessive circulating levels of sex hormones is the exogenous physiologic or pharmacologic intake of enteric and parenteric contraceptive hormonal devices.[8] The estrogen and progesterone oral contraceptive hormonal effect on bone morphogenesis and periodontal disease has been extensively studied.[3,4,7,9] Norplant[R], the newest contraceptive system, consists of subdermal contraceptive implants which continuously release a synthetic progestin, levonorgestrel, for five years.[10,11] To date, no *in vitro* studies have been reported examining the effects of levonorgestrel on cytokine production in bone morphogenesis. The specific aim of this study is to assay for the presence of IL-1β-stimulated IL-6 in bone cell culture supernatants treated with levonorgestrel and progesterone.

Materials and Methods

Primary Cell Culture

Cells were obtained from fetal murine calvaria. Using an aseptic technique, approximately 160 murine calvaria (*Mus mucularis*-Hsd: (ICR) BR) were collected. These were minced and the fragments obtained were enzymatically digested in 0.05% crude collagenase to dissociate cells from the bone matrix. The calvaria fragment/collagenase mixture was stirred for 20 minutes. The supernate

containing the freed bone cells was washed with 10 ml of Dulbecco's Modified Eagle medium (DMEM) and centrifuged for seven minutes at 1800 rpm. The supernate was discarded and the pellet resuspended with 10 ml Hank's Balanced Salts (HBSS). The mixture was again centrifuged for seven minutes at 1800 rpm. The supernate was discarded and the pellet resuspended in 4.5 ml DMEM. This suspension was then gently layered onto 4.5 ml of Ficoll-Paque. These steps were repeated for four digestions. The four suspensions on Ficoll-Paque were then centrifuged at 1650 rpm for 50 minutes. The buffy coats from the four digestions were combined and washed with HBSS and centrifuged at 1000 rpm for 10 minutes. The supernate was discarded and the pellet resuspended for a second wash in 14 ml of HBSS. Again the supernate was discarded and the pellet resuspended in a final 4 ml volume of DMEM.

Experimental Design

Cells were diluted with DMEM to deliver a concentration of 2 x 10^4 cells per well into 24-well ProNectin F coated plates (Protein polymer Technologies, San Diego, CA). The DMEM used was phenol red-free and contained 10% fetal bovine serum (FBS), non-essential amino acids, 15mM Hepes, Na Pyruvate, Penicillin (100 U/ml), Streptomycin (100μg/ml), Amphotericin B(0.5μg/ml), Gentamicin (10mg/ml) and 1,25 Dihydroxyvitamin D_3 (41.6μl/ml).

Cells were grown to confluence by day 5 at 37°C in 5% CO_2/95% ambient air and monitored for alkaline phosphatase activity throughout the experiment. To start the experiment, the medium was replaced with medium containing either control medium or medium containing levonorgestrel or progesterone on day 5. In addition, half of the samples included 10^{-10}M estrogen. Each steroid was dissolved in ethanol and further diluted in DMEM to a final ethanol concentration below 0.1%.

Control media contained the same ethanol concentration. Following incubation for 24 hours, all samples were stimulated with 0.2 ng/ml of IL-1β. Cultures were harvested on day 7 and growth media supernates were collected, stored at -70°C and assayed for the presence of IL-6. The remaining cell monolayer was solubilized with 0.03N NaOH and 1% sodium dodecyl sulfate and assayed for total protein with the Pierce (BCA) assay.

Steroids

To determine if there was a dose dependent response, the levonorgestrel was added to cell cultures at a final concentration of $5x10^{-9}$M, $5x10^{-8}$M, $5x10^{-7}$ M, $5x10^{-6}$M, which approximated high and low levels found in the plasma of women using NORPLANT^{R}. Progesterone was tested at 5×10^{-8}M. All of the samples were harvested and media changed in the same manner as those samples treated with levonorgestrel.

IL-6 Immunoassay

The IL-6 assay utilized a variation of the quantitative sandwich enzyme immunoassay method consisting of a monoclonal antibody specific for murine IL-6, a biotinylated polyclonal anti-murine antibody, an avidin-horse radish peroxidase conjugate, and substrate (PharMingen, San Diego, CA). Color detection was read at 405nm. A standard curve was generated with known concentrations of recombinant murine IL-6. Concentrations of unknown samples were extrapolated from the standard curve.

Alkaline Phosphatase Assay

The presence of alkaline phosphatase activity was detected using a commercially available test kit, Alkaline Phosphatase, Leukocyte kit (Sigma Diagnostics, St. Louis, MO). Briefly, cover slips of cells were fixed in a citrate-acetone-formaldehyde solution, stained with a diazonium salt solution (sodium nitrate/fast red violet alkaline solution/naphthol AS-BI solution), counter stained with hematoxylin and evaluated microscopically.

Data Analysis

The IL-6 and protein content for each sample were extrapolated from standard curves. Results were described as mg IL-6 per μg protein. These concentrations of treated (with levonorgestrel or progesterone) cell cultures versus non-treated (controls) cell cultures were compared by Kruskal-Wallis analysis of variance and differences among groups were analyzed by Duncan's multiple range test ($p < 0.05$). Cell cultures treated with levonorgestrel and progesterone in combination with estrogen were compared by the multivariant ANOVA ($p < 0.05$).

Results

Although one of the initial goals of this study was to develop a serum-free medium that could be used with the different concentrations of levonorgestrel and progesterone, the use of medium with reduced-serum failed to support osteoblast growth equivalent to that observed with 10% FBS. Therefore, the final DMEM/FBS medium was analyzed for the presence of hormones and growth factors. The levels of estradiol, progesterone, corticosterone, hydroxycorticosterone, insulin growth factor (IGF) binding protein, IGF-I/somatomedin-C, insulin, growth hormone, 17α-hydroxyprogesterone, testosterone, parathyroid hormone, insulin-like growth factor II, prolactin, cholesterol and estrone were found to be below normal physiologic levels of a woman in the follicular

phase of the menstrual cycle.

Protein concentration (μg/well) did not vary significantly among treatment groups ($p < 0.05$). Separate samples were microscopically observed for alkaline phosphatase with all samples indicating activity.

In the group of cultures not treated with estrogen, cells treated with levonorgestrel secreted significantly lower amounts of IL-1β-stimulated IL-6 versus control cells ($p < 0.05$)(Figure 1). Similarly, cultures treated with progesterone secreted significantly lower amounts of IL-1β-stimulated IL-6 versus control cells ($p < 0.05$), but significantly higher amounts than those treated with levonorgestrel ($p < 0.05$).

In the group of cultures treated with progesterone and levonorgestrel in combination with estrogen, there was a significant interaction between the hormones ($p < 0.05$) (Figure 2.). Specifically, at the highest level of levonorgestrel tested (5×10^{-6}M), IL-1β stimulated IL-6 production increased versus the lower concentrations of levonorgestrel ($p < 0.05$). Progesterone treated cells also showed a significant increase in IL-1β stimulated IL-6 production versus the lower levels of levonorgestrel ($p < 0.05$). There was no significant difference between the levonorgestrel (6 x 10-6M) and progesterone.

Discussion

Bone formation is accomplished through the process of cell proliferation, alkaline phosphatase expression, and matrix production mediated in part by osteoblasts. [12,13] These cells in turn regulate osteoclast activity through mediators such as sex hormones and cytokines.[14-16]

Cytokines have been shown to influence bone remodeling and periodontal pathogenesis through regulation of the immune response, hematopoiesis, and inflammation.[17] Interleukin-6 is produced by osteoblasts in response to local bone-resorbing agents, and induces bone resorption by stimulating osteoclastic activity.[18,19] Further, IL-6 mediates the anti-resorptive effect of estrogen, a major regulator and modulator of bone metabolism which has been shown to down-regulate the production of IL-6 by osteoblasts.[7,20-23] Progesterone modulates bone turnover as well as bone formation. Previous studies have shown that progesterone can also reduce IL-6 production by human gingival fibroblasts[24].

The most common source of exogenous steroid sex hormones is the administration of oral contraceptive pills in a combination of an estrogen and a progestin. Norplant contains only a synthetic progestin, levonorgestrel which is molecularly very similar to progesterone. Sustained levels of levonorgestrel produce a strong antiestrogenic effect on the body.[10] Due to the potential changes caused by the continuously elevated levels of this progestin, it was important to determine the effects of levonorgestrel on cytokine production.

Under the conditions of this *in vitro* bone cell model an attempt was made to duplicate the *in vivo* relationship of levonorgestrel with osteoblasts. The murine osteoblasts grown in these calvarial cultures were phenotypically and morphologically characteristic of an osteoblast. In the treatment groups without estrogen there was a significant inhibition of osteoblast secreted IL-6 after treatment with two different progestins. This effect agrees with previous studies in gingival fibroblasts indicating a progesterone mediated decrease in the expression of IL-6 mRNA versus non-treated controls.[24] In addition, levonorgestrel treated cell cultures secreted significantly less IL-6 than those treated with progesterone.

In the treatment groups that included estrogen, there was a combined effect of the estrogen with the two progestins. Interleukin-6 secretion increased for both the progesterone treated cells and the highest concentration of levornorgestrel ($5x10^{-6}M$) treated cells versus non-treated cells. This effect suggests a possible mediation of the individual effect of the three hormones when combined at commonly achieved human physiologic levels.

CONCLUSION

Certainly in the human body there is a complex interaction between hormones, bone morphogenesis and the immune system. These results, if applicable to the *in vivo* osteoblast environment, suggest that both levonorgestrel and progesterone when used in combination with estrogen may partially inhibit the anti-resorptive effect of estrogen through an increase in IL-1β stimulated IL-6 secretion. The implication of this study dictates that future *in vivo* studies be undertaken to examine the clinical and treatment significance of these hormone interactions.

Acknowledgments

The authors thank Norma Best for her excellent technical assistance and we also thank Dr. Dennis Runyan for his assistance with the statistical calculations. This research was funded by the U. S. Army Medical Research and Materiel Command, grant # LD1M5544.

Disclaimer

The views of this article are those of the author and do not reflect official policy or position of the United States Army or the Department of Defense.

References

1. Genco, RJ. Host responses in periodontal diseases: current concepts. J Perio. 1992; 63:338-355.

2. Horowitz, MC. Cytokines and estrogen in bone: anti-osteoporotic effects. Science. 1993; 260:626-27.

3. Vittek, S; Kirsch, S; Rappaport, M; Bergman, M and Southren, AL. Salivary concentrations of steroid hormones in males and cycling and postmenopausal females with and without periodontitis. J Perio Res. 1984; 19:545-55.

4. Miyagi, M; Hitoshi, A; Masayuki, Mand Iwamoto, Y. Effects of sex hormones on chemotaxis of human peripheral polymorphonuclear leukocytes and monocytes. J Perio. 1992; 63:28-32.

5. Arafat, AH. Periodontal status during pregnancy. J Perio. 1974; 45:641-43.

6. Rembiesa R, Ptak W, and Bubak M. The immunosuppressive effects of mouse placental steroids. Experimentia. 1974; 30:82-83.

7. Girasole, G; Jilka, RL; Passeri, G; Boswell, S; Boder, G; Williams, DC; Manolagas, SC. 17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens. J Clin Invest. 1992; 89(3):883-91.

8. Speroff, L; et al. Clinical Gynecologic Endocrinology and Infertility 4th Ed. Carol-Lynn Brown, ed. 1989. Williams and Wilkins.

9. Scheven, BA; Damen, CA; Hamilton, NJ; Verhaar, HJ; Duursma, SA. Stimulatory effects of estrogen and progesterone on proliferation and differentiation of normal human osteoblast-like cells in vitro. Biochem & Biophys Res Comm. 1992; 186(1):54-60.

10. Croxatto, HB. Norplant: levonorgestrel-releasing contraceptive implant (Review). Annals of Medicine. 1993; 25(2):155-60.

11. Brache, V; Alvarez-Sanchez, F; Faundes, A; Tejada, AS; Cochon,L. Ovarian endocrine function through five years of continuous treatment with Norplant subdermal contraceptive implants. Contraception. 1990; 41(2);169-77.

12. Stashenko, P; Obernesser, MS; and Dewhirst, FE. Effect of immune cytokines on bone. Immunological Investigations. 1989; 18(1-4):239-249.

13. Bonucci, E; Silvestrini, G; and Bianco, P. Extracellular alkaline phosphatase activity in mineralizing matrices of cartilage and bone: ultrastructural localization using a cerium-based method. Histochemistry. 1992; 97:323-27.

14. Dziak, R. Biochemical and Molecular Mediators of Bone Metabolism. J Perio. 1993; 64:407-415.

15. Heersche, J and Aubin, JE. Bone Volume I: the Osteoblast and Osteocyte. Brian K. Hall, ed, The Teleford Press, Inc., 1990.

16. Osdoby, P; Oursler, MJ and Anderson, F. The osteoblast and osteoclast cytodifferentiation. Progress in Developmental Biology, Part B. pp409-414. Alan R. Liss, Inc, 1986.

17. Akira, S; Hirano, T; Taga, T; Kishimoto, T. Biology of multifunctional cytokines: IL-6 and related molecules(IL-1 & TNF). FASEB J. 1990; 4:2860-67.

18. Mundy G. Role of cytokines in bone resorption. J Cellular Biochemistry. 1993; 53:296-300.

19. Ishimi Y, Miyaura C, Jin CH, Akatsu T, Abe E, Nakamura Y, Yamaguchi A, et al. IL-6 is produced by osteoblasts and induces bone resorption. 1990; 145(10):3297-303.

20. Hughes FJ and Howell GL. Interleukin-6 inhibits bone formation in vitro. Bone & Mineral. 1993; 21(1):21-8.

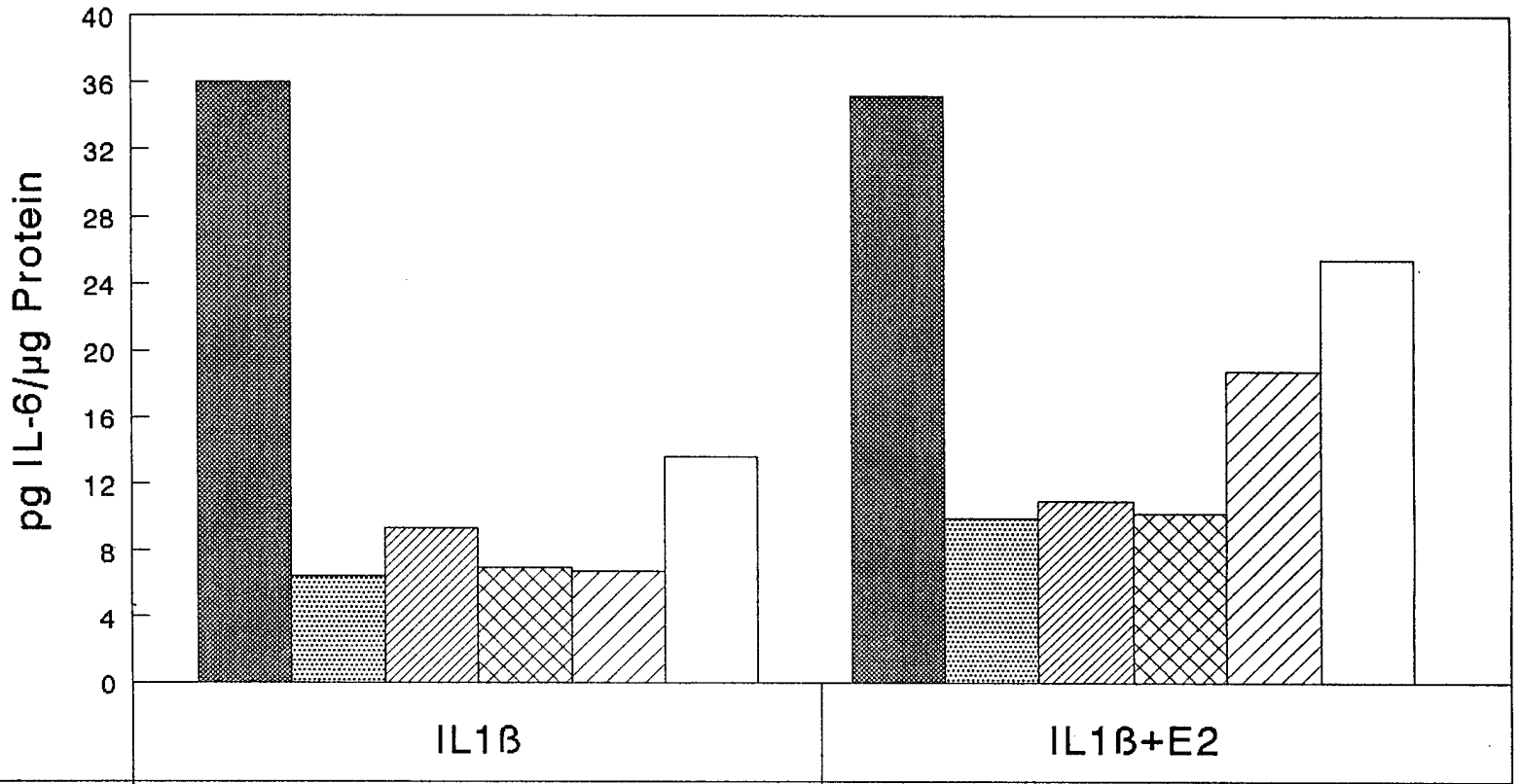
21. Purohit A, Flanagan AM, and Reed MJ. Estrogen synthesis by osteoblast cell lines. Endocrinology. 1992; 131(4):2027-9.

22. Chaudhary LR, Spelsberg TC, Riggs BL. Production of cytokines by normal human osteoblast-like cells in response to IL-1 beta and TNF-alpha: lack of regulation by 17β estradiol. Endocrinology. 1992; 130(5):2528-34.

23. Kalu, DN. Proliferation of tartrate-resistant acid phosphate positive multinucleate cells in ovariectomized animals. Proc Soc Exp Biol Med. 1990; 195:70-74.

24. Lapp CA, Thomas ME, and Lewis JB. Modulation by Progesterone of Interleukin-6 Production by Gingival Fibroblasts. J Periodontol. 1995; 66:279-284.

IL-6 Production by Murine Bone Cells



	IL1ß	IL1ß+E2
Control	36	35.2
5x10-9M Levo	6.5	9.95
5x10-8M Levo	9.38	11
5x10-7M Levo	7	10.25
5X10-6M Levo	6.78	18.83
5X10-8M Prog	13.6	25.45

FIGURE 1

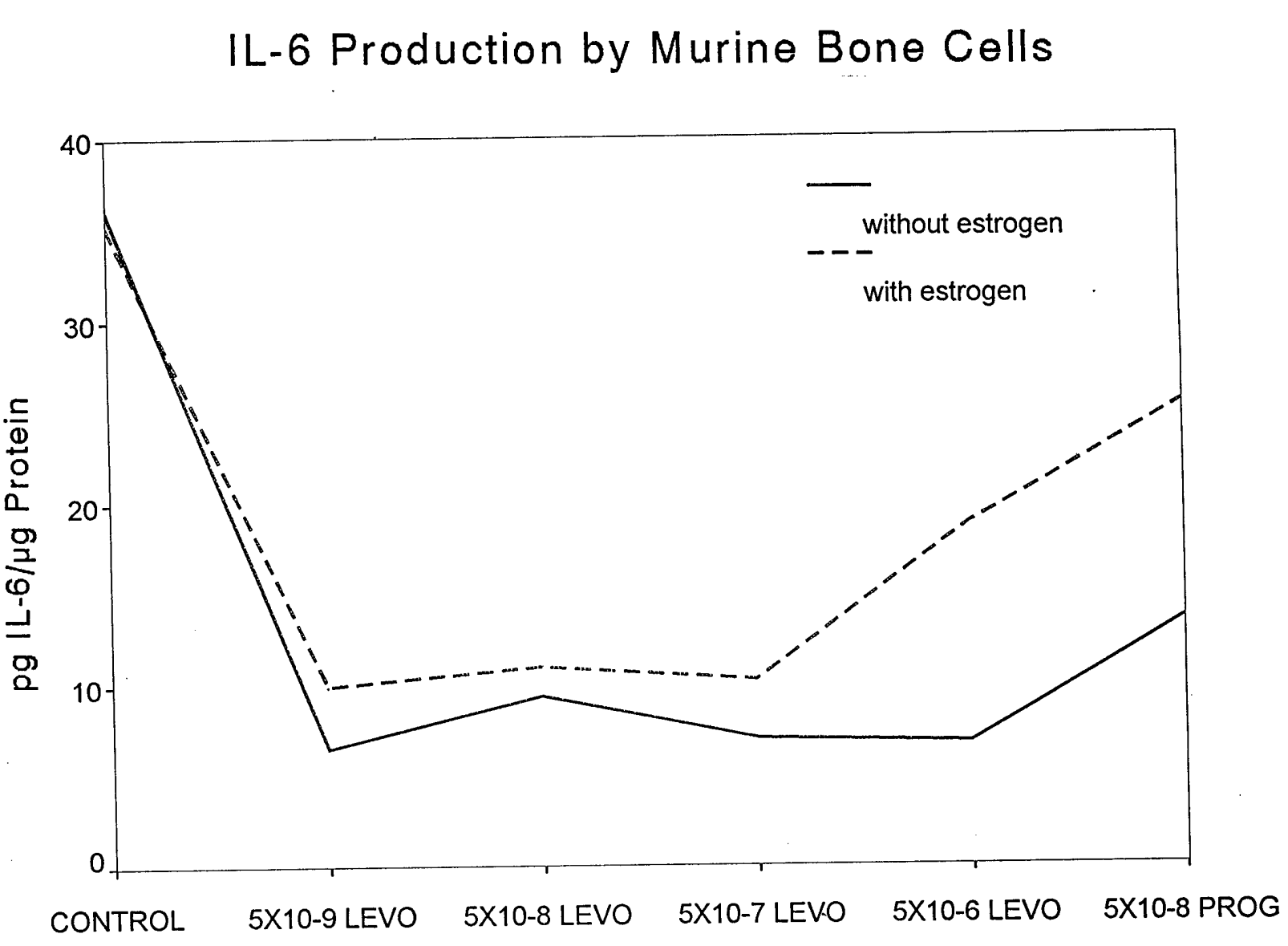

IL-6 Production by Murine Bone Cells
40
30
20
10
0
pg IL-6/µg Protein
without estrogen
with estrogen
CONTROL
5X10-9 LEVO
5X10-8 LEVO
5X10-7 LEVO
5X10-6 LEVO
5X10-8 PROG

FIGURE 2